UPTAKE AND ASSIMILATION OF AMINO ACIDS BY PLATYMONAS[1]

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Many algae, including some marine phytoplankters, behave heterotrophically in laboratory culture. Organic compounds have been systematically employed in algal culture since 1905 (Treboux). Glucose, pyruvate, and acetate commonly support or augment growth. Amino acids may serve as acceptable nitrogen sources in culture (Algeus, 1948; Arnow *et al.*, 1953; Ghosh and Burris, 1950; Pintner and Provasoli, 1963).

Such compounds are typically supplied in culture media at concentrations which are several orders of magnitude higher than concentrations which are possible in the normal environment. Studies of organic micronutrients provide a clear exception to this statement. Culture of algae can be an extremely sensitive technique for determination of vitamin B_{12} and other compounds in natural waters (Hutner and Provasoli, 1964). However there has not been comparable laboratory investigation of the possibility that part of the requirement for carbon and nitrogen may be met heterotrophically under normal conditions.

Heterotrophy has been discussed repeatedly as a possible factor in algal blooms (*e. g.*, Provasoli, 1960). Also, recent evidence has been presented for the existence of marine phytoplankters in the aphotic zone which may survive heterotrophically. Kimball *et al.* (1963) collected phytoplankton in oceanic aphotic zones, and confirmed the presence of chlorophyll *via* red fluorescence. Large numbers of viable unicellular flagellates have been collected throughout the aphotic regions of the north Atlantic (Fournier, 1966). Further, diatoms obtained in abyssal mud samples contained protoplasm and could be cultured at high pressures. Wood (1956) strongly supports the conclusion that these diatoms are autochthonous.

An ecologically meaningful investigation of uptake and assimilation of organic compounds must take account of very low naturally occurring concentrations. Amino acids were selected partly because recent information is available concerning levels in marine and estuarine waters. Langley Wood (1965) has reported free amino acid concentrations of 1 to 3 μmoles/liter. Similar values have been published by other authors (Chau and Riley, 1966; Degens *et al.*, 1964). Also, accumulation of amino acids has been reported in a wide variety of soft-bodied invertebrates (Stephens, 1963, 1964, 1967). Comparisons with algae may be informative. Finally, nitrogen is often an apparent limiting factor in algal populations. Naturally occurring free amino acids may contribute to the nitrogen needs of these organisms.

Platymonas was chosen as an experimental organism after preliminary observations demonstrated that it accumulated glycine very rapidly from dilute solution.

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It is occasionally abundant in phytoplankton (Margaleff, 1946) and is also found in high concentration in tidepools (Lewis and Taylor, 1921).

Materials and Methods

A culture of *Platymonas* was obtained from Dr. H. A. Lowenstam at the California Institute of Technology in the fall of 1965. The organism has been identified as *P. subcordiformis* (Hazen, 1921) on the basis of taxonomic characters listed by Kylin (Margaleff, 1946). Constant-volume 1-liter cultures were maintained at 28° C. in a medium containing 2×10^{-3} *M* KNO_3, 2×10^{-4} *M* K_2HPO_4, 10^{-5} *M* $FeCl_3$, and 0.001% EDTA in artificial sea water (Instant Ocean, Aquarium Systems Inc., Ohio). Sixteen hours of light per day were provided by cool white fluorescent fixtures. Cultures were vigorously aerated with 5% CO_2 in air. The pH varied from 6.0 to 7.5. Cells were also successfully grown on 2% agar made up in the culture medium. Liquid cultures were inoculated from sterile agar colonies to minimize bacterial contamination. Cell numbers in liquid culture were routinely determined colorimetrically. A standard curve was prepared relating optical density at 4000 Å to hemacytometer counts of formalin-fixed cells.

For measurement of uptake at low substrate concentration, amino acids uniformly labeled with C^{14} were added to a suspension of *Platymonas*. Cells were harvested in the log phase of growth, washed, and resuspended in filtered artificial sea water. Substrate concentrations greater than 10^{-6} molar were obtained using C^{12} amino acids to which tracer amounts of C^{14}-labeled material were added. Suspensions were incubated on a shaker bath at 28° C. One-ml. samples were collected on Millipore membranes (HAWP, 0.45 μ) at timed intervals and washed with 10 ml. artificial sea water. The dry filters were glued to aluminum planchets, and the radioactivity was measured using a thin-window gas flow detector. The radioactivity in the medium retained by the filter was about 1% of the activity found in the cells after an hour.

The disappearance of activity from the medium was also measured. Samples of the filtrate were dried on aluminum planchets. It was found that dilution of these filtrate samples with 19 volumes of distilled water gave an effective sample thickness equal to that of the cells retained on the filters. Consequently this procedure was adopted to facilitate comparison of radioactivity levels in the cells and in the medium (Fig. 1).

Results

Amino acid uptake

Growth curves for cultures supplied with three nitrogen sources are presented in Figure 2. Equivalent amounts of nitrogen provided as glycine or as potassium nitrate supported growth equally well. The doubling time in the log plase was approximately 22 hours. It is apparent that the same weight of nitrogen supplied as ammonium phosphate supported growth only after a longer lag period.

A number of amino acids were accumulated by *Platymonas* from very dilute solution. Table I lists the percentage of radioactivity removed from the medium by 2×10^6 cells/ml. after 30 minutes. Amino acids representing major chemical groups (basic, acidic, aliphatic, aromatic) were accumulated at comparable rates.

Only negligible amounts of glucose got into the cells. Uptake rates were directly proportional to cell numbers (Table II) in the range tested. Five $\times 10^5$ cells/ml. gave linear uptake for about 30 minutes and was selected as a standard cell concentration for further studies.

Bacteria were occasionally visible with the microscope in older cultures, but control experiments, utilizing heavy doses of antibiotics (neomycin, streptomycin, penicillin G at 100 mg./l.), and repeated washing of cells produced no decrease in uptake. In addition, autoradiography of cell suspensions exposed for a brief period to H^3-glycine demonstrated that the radioactivity was associated with *Platymonas* cells.

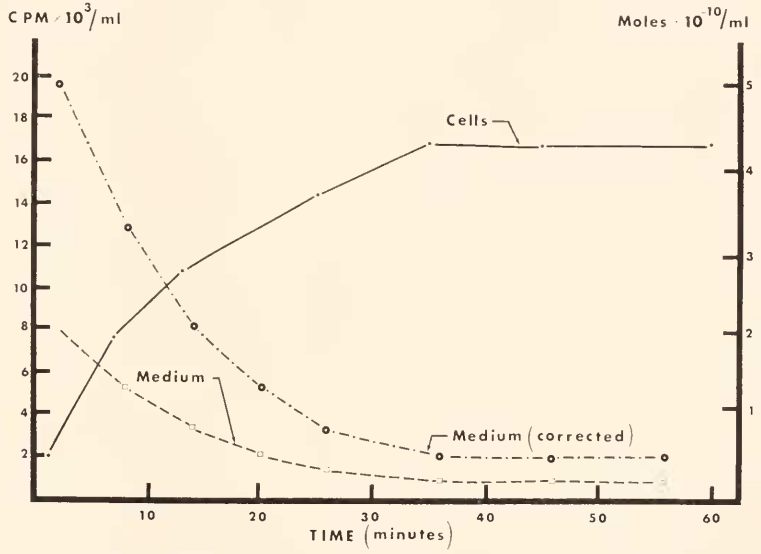

FIGURE 1. Uptake of radioactive glycine by *Platymonas* and concomitant disappearance of radioactivity from the medium. Cell concentration $= 4 \times 10^5$ cells/ml., glycine $= 5 \times 10^{-7}$ *M*. [⊙] CPM/ml. cells. [□] CPM/ml. medium. [○] CPM/ml. medium diluted with distilled water to reduce apparent sample thickness (see text). CPM in cell samples [⊙] and corrected medium samples [○] are converted to moles glycine (right ordinate).

Amino acid uptake at high concentration was demonstrated by following the concentration in the ambient medium colorimetrically. The ninhydrin technique used was that of Clark (1964) as described by Stephens and Virkar (1966). When glycine was supplied at 10^{-4} moles/liter in a suspension of 2×10^6 cells per ml., 67% was removed in one hour. After cells had accumulated labeled amino acid, incubation in fresh medium for 4 hours did not leach out activity significantly above background.

Figure 3 illustrates typical uptake curves at four concentrations of glycine. Slopes (uptake velocity) were determined using the least squares method for 13 such curves at ambient concentrations ranging from 10^{-4} to 1.4×10^{-6} molar glycine. A plot of the reciprocal of uptake velocity against the reciprocal of ambient con-

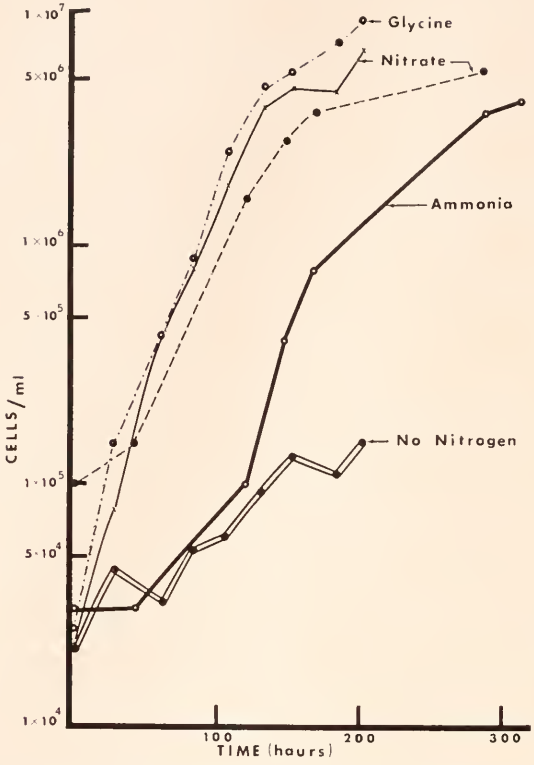

FIGURE 2. *Platymonas* growth curves. Equivalent weights of nitrogen (30 mg./l.) as glycine, $K NO_3$, and $(NH_4)_2 HPO_4$ were supplied. Cell number was determined colorimetrically.

centration is presented as Figure 4. This is equivalent to a Lineweaver-Burk plot and permits estimation of maximum velocity from the intercept of the line. The apparent maximum velocity is 1.3×10^{-10} moles of glycine/minute/5×10^5 cells. The straight line suggests that the rate of uptake is limited by an absorptive step at high concentrations. The process of accumulation of radioactivity in the form

TABLE I

Uptake of amino acids by suspensions of 2×10^6 cells per ml. Per cent of substrate accumulated after 30 minutes is shown at substrate concentrations ranging from 1×10^{-6} to 2×10^{-7} molar

	% Accumulation
Arginine	>95
Glutamic acid	65
Glycine	>95
Phenylalanine	50
Valine	90
Glucose	4

TABLE II

Uptake rates of glycine at three cell concentrations. Cells were exposed to 7×10^{-7} M glycine-C^{14} for 30 minutes

Cell concentration (number per ml.)	Ratio	Uptake rate (cpm/min.)	Ratio
3×10^5	1	90	1
1×10^6	3.3	270	3.0
2.6×10^6	8.7	775	8.6

of glycine-C^{14} continued at concentrations as low as 5×10^{-8} molar; a suspension of 10^6 cells per ml. removed 96% of the radioactivity after 60 minutes.

Assimilation

Cells incubated with glycine-C^{14} were collected at intervals and extracted with 80% ethanol. The extract was evaporated to dryness and the residue dissolved in 10% isopropanol. Ten to 20 microliters of this solution were spotted on Whatman #1 filter paper. An ascending separation was carried out in 2 dimensions with the solvent systems: butanol, acetic acid, water, 12:3:5, v/v/v; phenol, water 18:5, w/v. Autoradiograms (Kodak "No-Screen" medical x-ray film) of resulting

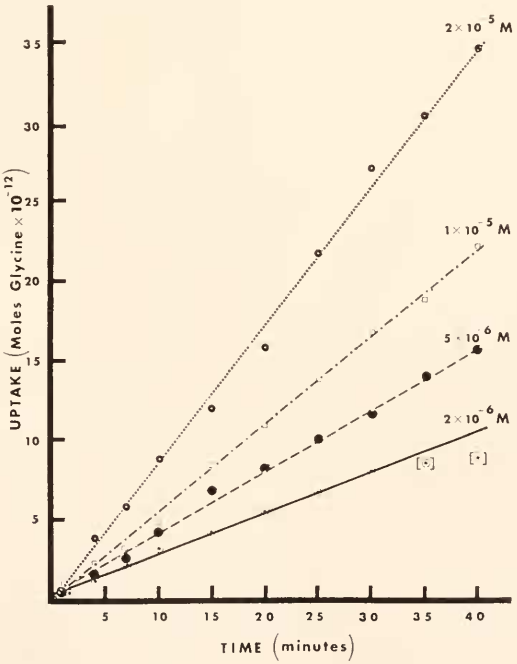

FIGURE 3. Uptake of glycine-C^{14} at 4 concentrations. Points are 1-ml. samples containing 5×10^5 cells. Lines obtained with the least squares method. [⊙] indicates decrease in uptake rate due to depletion of glycine in the medium.

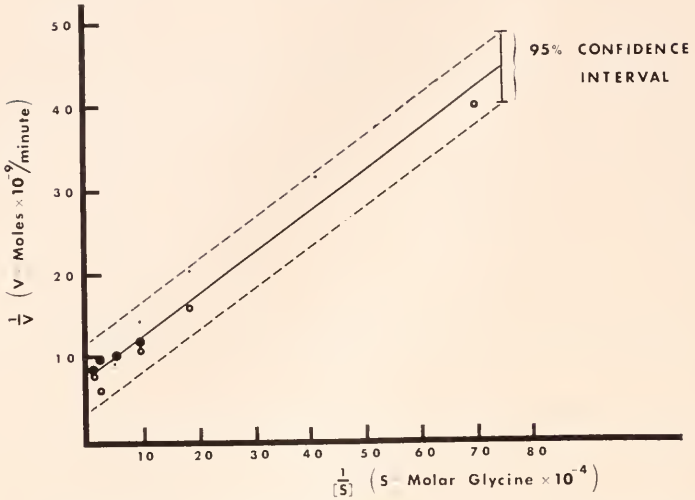

FIGURE 4. Double reciprocal plot relating uptake velocity to ambient glycine concentration. Each point represents a rate determined from curves like those in Figure 3. ⊙, ●, ○, are from separate cultures. The line was fitted by the least squares method.

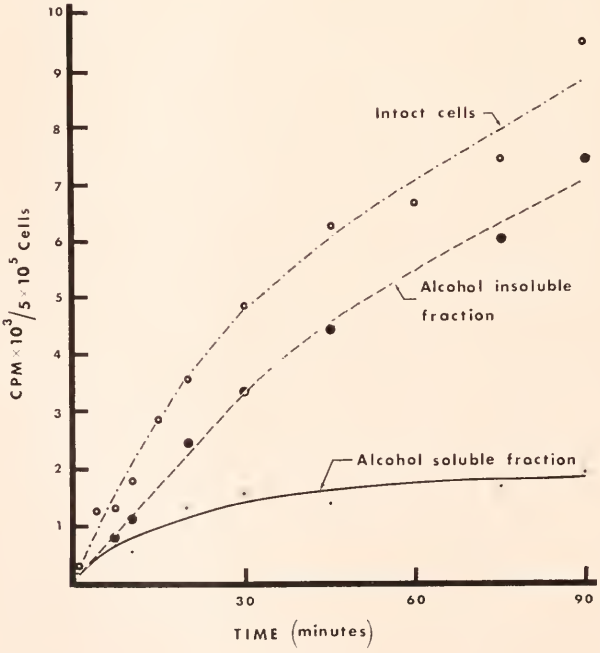

FIGURE 5. Distribution of radioactive glycine (4×10^{-7} *M*). [○] Total radioactivity in intact cells. [⊙] CPM in alcohol extract. [●] Radioactivity remaining in cell residue (alcohol-insoluble fraction).

chromatograms were exposed for one week. The chromatogram was sprayed with ninhydrin reagent to identify amino acids. After 5 minutes of incubation, most of the alcohol-soluble radioactivity in the cells was present as glycine (identified by co-chromatography of an authentic sample). After 60 minutes incubation, most of the radioactivity appeared as three different spots although glycine-C^{14} was still present. The spots were not identified.

Radioactivity in cells incubated with glycine or phenylalanine was separated into alcohol-soluble and alcohol-insoluble fractions. Radioactive cells were collected on Gelman (Acropore, alcohol-insoluble) filters. Activity was measured in intact cells before extraction with 10 ml. of 80% ethanol for 10 minutes. Radioactivity in 0.5-ml. aliquots of the alcohol was determined. Finally, radioactivity remaining on filters (the alcohol-insoluble fraction) was measured. Figure 5 presents a typical set of observations. The alcohol-soluble fraction of the radioactivity accumulated by the cells rapidly reached a plateau. Continued uptake from the medium appeared to depend on assimilation into other compounds, presumably polypeptides or proteins.

Evolution of $C^{14}O_2$ was observed in cells suspended in 10^{-6} *M* glycine-C^{14}. $C^{14}O_2$ was identified qualitatively by a Conway diffusion technique. CO_2 was

TABLE III

Nitrogen content of cells as a function of age of culture

Culture	mg. N per 10^8 cells $n = 3$	Nitrogen % dry wt	Culture age
1	1.33 ± .02	7.1	log phase
2	1.15 ± .13	7.2	end of log
3	0.73 ± .05	4.7	past log phase

trapped in 10% KOH. Small amounts of labeled CO_2 could be found after 2 hours of incubation, indicating that the accumulated glycine was entering oxidative pathways.

Total cell nitrogen was determined with the semi-micro Kjeldahl technique described by Steyermark (1961). Dry weight of cells was found by collecting 10^7 cells on preweighed filters, washing quickly with distilled water, and drying to constant weight at 70° C. The resulting values (Table III) are consistent with published data for other green algae. The lowest value was found in the oldest culture.

DISCUSSION

Platymonas subcordiformis is capable of rapid accumulation and assimilation of all amino acids tested from very dilute solutions. Glycine and nitrate are equally effective nitrogen sources when supplied at high concentration (2×10^{-3} *M*). It is of interest to consider whether a significant fraction of the nitrogen requirement of the cells might be provided at ambient concentrations which obtain in nature.

At 1.0 micromole of glycine per liter, approximately 34×10^{-10} moles of glycine per minute per 10^8 cells can be accumulated. This uptake rate is derived from

Figure 4. The nitrogen taken up during the observed doubling time of 22 hours can be obtained from the following relation (*cf.* Fencl, 1966) where

$$U = \text{uptake in moles}$$

$$N = \text{cell number}$$

$$r = \text{specific growth rate (ln 2/doubling time)}$$

$$k = \text{uptake rate}$$

$$t = \text{time}$$

$$\frac{dU}{dt} = kN_0e^{rt}$$

$$U = \frac{kN_0}{r}\left[e^{rt_1} - e^{rt_0}\right]$$

For t_1 equal to the doubling time and t_0 equal to 0, this equation simplifies to $U = kN_0/r\ [2 - 1]$ or $U = kN_0/r$. Substituting measured values for r and k and taking N_0 as 10^8 cells, we obtain 0.091 mg. nitrogen. This is 7% to 13% of the nitrogen assimilated by the cells in culture (Table III). Hence, uptake from a glycine solution at 10^{-6} moles/liter provides for roughly 10% of the nitrogen requirement of the cells. This represents a minimum estimate of the probable contribution of free amino acids under natural circumstances. The estimate is based on cells in the log phase of growth with excess nitrate and optimum light conditions. The doubling time is probably longer in nature under usual circumstances. In addition these conditions maximize the nitrogen content of the cells. Fowden (1962) stated that nitrogen content in starved algae may decrease from 8% to less than 1%. Thus the probable contribution of free amino acids in the environment will be greater than was calculated above. Most or all of the required nitrogen might well be provided by this mechanism under selected natural conditions.

Algeus (1948) noted that some fresh-water algae could utilize amino acids as a nitrogen source. He determined the pH of cultures as they aged and concluded that the algae were deaminating the amino acids and the resulting ammonia was being used as a source of nitrogen. A mechanism of this kind is very unlikely in *Platymonas.* Chromatographic evidence indicated that glycine does enter the cells. Repeated efforts to demonstrate ammonia in cultures incubated with glycine concentrations of 10^{-4} moles/liter for a period of 24 hours were negative. Also ammonium phosphate supplied to these cells was utilized only after a considerable lag period (Fig. 1) although a possible inhibiting effect of ammonium at high concentrations was not investigated.

Sloan and Strickland (1966) studied the uptake of several organic compounds by algae and found that the diatom, *Thalassiosira,* removed glutamate from solution more effectively than glucose or acetate. It is impossible to make a direct comparison of their results with ours since they discarded the uptake during the first hour as a blank. However, the rates they report are lower than those measured

for *Platymonas* by at least an order of magnitude. There is little additional information available on the distribution of this capacity among phytoplankters but we are preparing a report indicating rapid uptake and assimilation in several genera of intertidal macroscopic algae.

The uptake and assimilation of amino acids by *Platymonas* differs from that reported for animals by Stephens (1967) in two respects. Soft-bodied marine invertebrates typically have large free amino acid pools and show much slower rates of uptake into the alcohol-insoluble fraction of the organism. Also no rapid conversion among compounds in the alcohol-soluble fraction such as that reported here has been detected.

It has been assumed that short-term measurements presented here are valid over extended periods. Uptake of glycine has been observed qualitatively for periods as long as 20 hours. Reliable quantitative data for periods longer than 2 to 4 hours have not been obtained.

The discussion has been presented in terms of nitrogen but a similar calculation could be adduced for organic carbon. Cell carbon was not measured but if a figure of 30% is accepted from the literature (Wimpenny, 1966), the contribution of organic carbon by uptake from 10^{-6} molar solutions of amino acid is substantial. Assuming a reasonable mixture of amino acids in natural circumstances, 10% of cell carbon is provided during log phase growth. Higher fractions can be speculatively defended under less ideal conditions as has been suggested above.

It seems more reasonable to consider this mechanism in relation to nitrogen requirements in photosynthetic cells. In any case, the uptake of amino acids is not influenced by the presence of nitrate in medium. There is no modification of the uptake rate of labeled glycine in the presence of potassium nitrate at concentrations 100 to 1000 times greater than that of the amino acid. Consequently, as pointed out by E. J. F. Wood (1965), studies which assume inorganic nitrogen to be equivalent to the total nitrogen available for plant productivity should be treated with reserve.

In conclusion, the evidence for a normal role of heterotrophy in the economy of at least some algae is now quite substantial. In particular, *Platymonas* can obtain significant amounts of nitrogen and carbon from ambient concentrations of amino acids which lie in the normal range observed in the habitat of this organism.

Summary

The marine flagellate *Platymonas subcordiformis* rapidly takes up amino acids at concentrations likely to be found in nature. The relation between velocity of uptake and substrate concentration was determined, and the apparent maximum uptake velocity estimated. Accumulated C^{14} amino acids were assimilated into alcohol-insoluble compounds and entered oxidative pathways as shown by detection of evolved $C^{14}O_2$. The contribution of the uptake mechanism at an ambient glycine concentration of 1.0 μmole/l. to nitrogen requirement of the cell was found to be roughly 10% for optimal laboratory growth conditions. In nature, where growth conditions are probably less favorable, the contribution would be considerably greater. This provides further support for a normal role of heterotrophy in phytoplankters.

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